



Research Paper

Transforming Growth Factor- β 1 as a Predictor for the Development of Hepatocellular Carcinoma: A Nested Case–Controlled Study



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ABSTRACT

Background: Transforming growth factor- β 1 (TGF- β 1) reportedly acts as a tumor suppressor in tumorigenesis. However, little is known as to how TGF- β 1 concentrations change prior to the development of hepatocellular carcinoma (HCC) in humans. We examined the association between the serum TGF- β 1 concentrations and death from HCC to determine whether the serum TGF- β 1 can be a predictor of incident HCC.

Methods: We conducted a nested case–controlled study of participants in the Japan Collaborative Cohort Study for Evaluation of Cancer Risk. We used a conditional logistic regression analysis to estimate the adjusted relative risks (aRRs) of death from HCC according to the serum TGF- β 1 concentrations among 1940 participants including 83 patients with HCC and 1857 controls matched for age, sex, and hepatitis C virus (HCV)-antibody seropositivity. **Findings:** When serum TGF- β 1 was modelled as a continuous variable, the aRR of death from HCC associated with a decrement of 7.9 ng/mL (one standard deviation) in the serum TGF- β 1 concentrations was 2.3 (95% CI 1.7–3.0, $P < 0.001$) for all the subjects. The area under the receiver operating characteristic curve for the serum TGF- β 1 concentrations was 0.78 ($P < 0.05$).

Interpretation: Our finding suggests that TGF- β 1 serves as a predictor for HCC.

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1. Introduction

Transforming growth factor- β 1 (TGF- β 1) is a member of the TGF- β family, which regulates many cell functions such as differentiation, immune homeostasis, and carcinogenesis (Massagué et al., 2000). Reportedly, TGF- β 1 has a dual nature—it acts as a tumor suppressor at early-stage carcinogenesis but promotes invasion and metastasis at later stages (Derynck et al., 2001; Ikushima and Miyazono, 2010; Lin et al., 2015). However, little is known as to how TGF- β 1 concentrations change prior to the development of hepatocellular carcinoma (HCC) in humans.

HCC is the third most common cause of cancer death worldwide (Ferlay et al., 2010). HCC has a poor prognosis, with an overall five-year mortality rate of >88% (Mittal and El-Serag, 2013). Current curative

treatments such as surgical resection are limited to early-stage HCC; however, HCC is difficult to diagnose early enough to perform a radical treatment (El-Serag et al., 2008; Imamura et al., 2003). Although several biomarkers have been proposed for early detection of HCC, such as alpha-fetoprotein (AFP), *Lens culinaris* agglutinin reactive AFP (AFP-L3), des- γ -carboxy prothrombin (DCP), glypican-3 (GPC3), and Golgi protein 73 (GP73) (Malaguarnera et al., 2010; Yamamoto et al., 2010), no biomarker has been known to become elevated even before development of HCC so far. Discovery of such biomarker is warranted to identify patients who are at higher risk of developing HCC. In general, to the best of our knowledge, there has been no serum biomarker that can predict the development of malignancy even before malignant cells are formed. As TGF- β 1 is shown to function as a tumor suppressor when cancer emerges, TGF- β 1 would be a potential candidate as a predictor for HCC. Thus far, no prospective cohort study has assessed the relationship between the serum TGF- β 1 concentrations and incidence of HCC in the general population.

Therefore, in the current nested case–controlled study, we examined prospective association between serum TGF- β 1 concentrations and the

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risk of fatal HCC to investigate whether the serum TGF- β 1 can be used as a predictor of incident HCC.

2. Materials and Methods

2.1. Study Population and Serum Samples

We conducted a nested case-controlled study within the Japan Collaborative Cohort Study (the JACC Study) for Evaluation of Cancer Risk. The baseline survey for this study was performed in 1988–1990. A total of 110,585 participants (46,395 men and 64,190 women) aged 40–79 years and living in 45 areas across Japan responded to self-administered questionnaires regarding their lifestyles, such as their smoking and alcohol drinking status, and their medical histories of major diseases, including liver diseases and cancer. The JACC Study was approved by the Ethical Boards at the Osaka University School of Medicine and the Nagoya University School of Medicine, and participants' informed consent was obtained.

Participants underwent municipal health screening examinations where peripheral blood samples were collected from 39,242 individuals. Serum from these samples was separated and stored at -80°C . This has been proven to be a reliable method to maintain TGF- β 1 concentrations because there was no significant difference in measured values of TGF- β 1 between newly collected serum and frozen specimens that had been stored for nine years (Ito et al., 2005; Tamakoshi et al., 2009).

2.2. Case Ascertainment

We followed the subjects from the date of the baseline survey through December 31, 1999 to obtain information on the cause of death from death certificates provided by the Ministry of Health, Labour and Welfare in Japan. We identified liver cancer according to the International Classification of Diseases, tenth revision (ICD-10) code C22.0–22.9.

2.3. Surveillance

For the mortality surveillance, we reviewed death certificates and classified the underlying causes of death coded for the National Vital Statistics according to ICD-10. All deaths that occurred in the cohort were ascertained by death certificates from public health centers, except for participants who died after moving from their original areas, which became censored cases.

2.4. Measurements of Serum Constituents

We measured the constituent serum concentrations, such as TGF- β 1, among all the cancer patients as well as their healthy controls. The serum TGF- β 1 concentrations were measured using a quantitative sandwich enzyme immunoassay, with a commercially available kit (Quantikine; R&D Systems Inc. Minneapolis, MN, USA) at SRL Laboratory (Hachioji, Tokyo, Japan) in 1999 (Ito et al., 2005; Tamakoshi et al., 2009).

2.5. Statistical Analysis

The statistical analyses were based on the mortality during the follow-up period (1989–1999). Differences in the mean values of the baseline characteristics between the HCC and control groups were tested using the Student *t*-test. We examined the association between serum TGF- β 1 concentrations and mortality from HCC.

Age-adjusted and multivariate-adjusted relative risks (aRRs) and their 95% confidence intervals (CIs) of HCC-related mortality stratified by hepatitis C virus (HCV)-positivity were calculated after adjustments for age and potential confounding factors using a conditional logistic

regression analysis. The confounding variables included age (years), smoking (never, former or current smokers), drinking habits (never, former, or current drinkers who consumed <20 g or ≥ 20 g of ethanol per day), and history of diabetes mellitus or liver disease. These factors are known to increase the risk of HCC (Forner et al., 2012). Although body-mass index >40 is known to be a risk factor for mortality (Forner et al., 2012), we did not adjust for body-mass index because no participant had body-mass index >40 in our population. We performed HCV tests in the current study because about 80% of all HCC cases in Japan are thought to be associated with HCV infection (El-Serag et al., 2001).

In order to exclude the possibility of undetected HCC at baseline when the TGF- β 1 concentrations were measured, we performed a sensitivity analysis limiting the patient population who survived for at least five years after starting follow-up given a median five-year survival rate of 6% in patients with HCC (Siegel and Massagué, 2003).

For the trend analysis, we calculated aRRs per one standard deviation (SD) decrease in the TGF- β 1 concentrations as a continuous variable. Receiver operating characteristics (ROC) curves were constructed to assess the areas under the curve (AUC) and the 95% CI. We calculated the cut-off point by the Euclidean distance method. All the analyses were performed using SAS statistical package version 9.2 (SAS Institute Inc., Cary, NC). A two-sided *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Description of the Study Population

Every participant in the study was followed for ten years. During the follow-up period, 550 deaths from liver cancer were observed. Baseline serum samples had been collected from 120 out of these 550 subjects. We excluded 24 cases that did not have a C22.0 code or that had insufficient data regarding HCV positivity. Out of the 96 eligible patients who had died from HCC (ICD-10 code C22.0) as of the endpoint of the current study, 13 patients did not have serum data for TGF- β 1. Among the remaining 83 patients, 58 (70%) tested positive for HCV and 25 (30%) tested negative. As potential controls, serum samples from 10,574 subjects living in the same geographical areas as the cases were also screened for HCV infection. After excluding those with missing TGF- β 1 data, 491 (9%) of the controls were HCV-positive, and 4826 (91%) were HCV-negative. We chose as many controls per case as possible, matching for age (same five-year strata), sex, and HCV seropositivity. Finally, the control group consisted of 232 HCV-positive controls (four controls per case) and 1625 HCV-negative controls (65 controls per case). Therefore, we identified 83 deaths due to HCC and 1857 age- and sex-matched controls.

3.2. Comparison Between Cases and Controls at Baseline

The HCC cases and controls were well matched for age and sex in both the HCV-positive and the HCV-negative groups (Table 1). The mean age was 63.9 years in the HCC group and 64.8 years in the control group. The HCC group was more likely to smoke and drink than the controls and to have histories of liver disease and diabetes mellitus. The serum TGF- β 1 concentrations were lower in the HCC group (26.5 ng/mL) than in the control group (35.9 ng/mL; $P < 0.001$).

3.3. Association Between Serum TGF- β 1 Concentration and the Risk of Incident HCC

Table 2 shows the relative risks for death from HCC according to one SD decrement of the TGF- β 1 concentrations (7.9 ng/mL). Lower TGF- β 1 concentrations were associated with a higher risk of death from HCC in an unadjusted model; each SD decrement in the serum TGF- β 1 concentration was associated with a 2.6-fold increase in the risk of death from

Table 1
Baseline characteristics of 83 patients with fatal hepatocellular carcinoma and 1857 controls.

Participant characteristics	Cases (n = 83)	Controls (n = 1857)	P
Mean age, years	63.9 ± 6.4	64.8 ± 6.1	0.19
Men	54 (65)	1131 (61)	0.45
Mean serum TGF-β1, ng/mL	26.5 ± 9.5	35.9 ± 7.9	<0.0001
Smoking			
Never smokers	25 (30)	869 (47)	0.003
Former smokers	14 (17)	324 (17)	0.89
Current smokers	36 (43)	572 (31)	0.02
Current drinking			
Never drinkers	29 (35)	784 (42)	0.19
Former drinkers	14 (17)	74 (4)	0.003
Current drinkers	36 (43)	935 (50)	0.21
Mean ethanol intake among current drinkers, g/day	12.8 ± 8.0	12.7 ± 9.2	0.98
History of diabetes mellitus	12 (14)	89 (5)	0.02
History of liver diseases	37 (45)	131 (7)	<0.0001

Data are shown as n (%) unless otherwise stated.

HCC. This association was more evident in the HCV-positive group than for the HCV-negative group. After adjusting for smoking, drinking habits, and prior history of diabetes mellitus and liver disease, the adjusted RRs were 2.3 (95% CI: 1.7–3.0, $P < 0.001$) for the total subjects, 3.2 (95% CI: 2.1–5.0, $P < 0.001$) for the HCV-positive group, and 1.6 (95% CI: 1.0–2.3, $P = 0.03$) for the HCV-negative group. There was a significant effect modification by HCV status (interaction $P = 0.02$). The sensitivity analysis restricting the patient population to those who survived for at least five years of follow-up showed similar results as the main analysis (Table 2).

3.4. Predictive Value of TGF-β1 According to HCV-Positivity

The area under the receiver operating curve (AUC-ROC) for the serum TGF-β1 concentration was 0.78 for predicting HCC-related death. The cut-off point was 30.0 ng/mL with the sensitivity of 0.70 and the specificity of 0.77 (Fig. 1). In the HCV-positive group, the AUC-ROC was 0.83 and the cut-off point was 29.3 ng/mL (sensitivity: 0.78; specificity: 0.75). As for the HCV-negative group, the AUC-ROC was 0.61, and the cut-off point was undetermined.

Table 2
Multivariate-adjusted relative risks (aRRs) and 95% confidence intervals (CIs) of fatal hepatocellular carcinoma cases according to 1-SD decrements in the serum TGF-β1 concentration.

	All subjects	HCV-positive	HCV-negative
Total subjects			
Cases n	83	58	25
Controls n	1857	232	1625
RR (95% CI, P value)	2.6 (2.1–3.4, <0.0001) [†]	3.6 (2.4–5.2, <0.0001) [†]	1.8 (1.2–2.7, 0.003) [*]
Multivariate aRR (95% CI, P value)	2.3 (1.7–3.0, <0.0001) [†]	3.2 (2.1–5.0, <0.0001) [†]	1.6 (1.0–2.3, 0.03) [*]
Subjects who survived for at least 5 years			
Cases n	62	43	19
Controls n	1407	172	1235
RR (95% CI, P value)	2.7 (2.0–3.6, <0.0001) [†]	4.3 (2.5–7.2, <0.0001) [†]	1.6 (1.0–2.5, 0.05)
Multivariate aRR (95% CI, P value)	2.3 (1.6–3.1, <0.0001) [†]	3.6 (2.1–6.4, <0.0001) [†]	1.4 (0.9–2.3, 0.17)

1-SD decrements in the serum TGF-β1 concentration = 7.9 ng/mL.

^{*} $p < 0.05$.

[†] $p < 0.0001$.

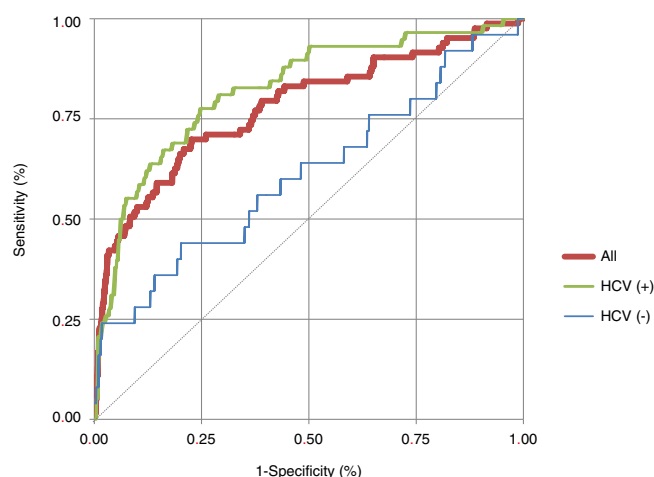


Fig. 1. Receiver operating curve (ROC) for the risk of hepatocellular carcinoma according to the serum TGF-β1 concentration. The area under the ROC (AUC-ROC) for the serum TGF-β1 concentrations was 0.78; the cut-off point was 30.0 ng/mL (sensitivity: 0.70; specificity: 0.77). In the HCV-positive group, the AUC-ROC was 0.83; the cut-off point was 29.3 ng/mL (sensitivity: 0.78; specificity: 0.75). In the HCV-negative group, the AUC-ROC was 0.61; the cut-off point was undetermined.

4. Discussion

The findings of this nested case-controlled study using a large real-world population-based dataset exhibited that low serum TGF-β1 concentrations predict death from HCC, with a 160% increase in the risk per one SD decrease in the TGF-β1 concentrations. This association remained statistically significant even after adjusting for confounding factors and in a sensitivity analysis restricted to participants who survived for at least five years after the baseline survey, effectively eliminating the possibility that these patients had undiagnosed HCC at the baseline survey. Our data demonstrates that low levels of TGF-β1 can identify patients who are at higher risk of developing HCC. Furthermore, the predictive value was significantly higher among HCV-positive patients. TGF-β1 may be a serum predictor that becomes altered well before the development of clinically detectable malignancy.

Our findings are in agreement with the general theory that TGF-β1 works as a tumor suppressor during tumorigenesis. TGF-β1 is frequently present in the tumor microenvironment and prevents premalignant progression during the beginning phases of carcinogenesis (Blobe et al., 2000; Derynck et al., 2001; Ikushima and Miyazono, 2010; Massagué et al., 2000). Many carcinomas have loss-of-function mutations in the TGF-β1 pathway that involve its receptors or downstream signal transducers such as SMADs (Tang et al., 2008). In terms of HCC, an experimental study of human HCC tissue has shown that HCC could arise from an IL-6-driven transformed stem cell with inactivated TGF-β signaling (Yoshizawa, 2002), implying that TGF-β1 has a tumor suppressor function. Therefore, it is reasonable to postulate that the blood TGF-β1 concentrations may be decreased before the emergence of HCC.

According to HCV status, we found a stronger association between low TGF-β1 concentrations and the risk of death from HCC among the HCV-positive group compared to the HCV-negative group, suggesting that the predictive value of TGF-β1 is more potent in higher risk HCV-positive individuals. This finding has important public health implications because this is the very patient population that consumes a large amount of healthcare resources, and therefore the development of strategies for early detection of HCC and prompt treatment is warranted. Our observations promote further investigation to assess whether these high-risk individuals with HCV infection and low TGF-β1 concentrations would benefit from more frequent surveillance with abdominal ultrasound and the measurement of biomarkers such as AFP, which are standard procedures for the early HCC detection (Forner et al., 2012).

The strengths of our study come from its large sample size and a long-term follow-up in the general population in the natural setting. The ten-year follow-up enabled us to perform sensitivity analysis in patients who survived at least for five years, for whom we could assume that these patients did not have HCC at baseline given the high five-year mortality among patients with HCC. This was effective for ensuring that we have collected blood samples to assess concentrations of serum biomarkers prior to the development of malignancy. Additionally, with the data on HCV seropositivity, we could examine associations between serum TGF- β 1 concentrations and HCC mortality stratified by HCV-positivity, revealing that the predictive value of TGF- β 1 is higher in HCV-positive patients. In contrast to the existing HCC biomarkers that become altered after the development of HCC, our data shows that TGF- β 1 concentrations can be disturbed even in the absence of HCC, thereby identifying high-risk patients who are more likely to develop HCC in the future.

Our study has several limitations. Although our cohort study had a large sample size, the number of HCC patients was limited because of the low HCC mortality rate among the participants, especially for the HCV-negative group. As only 25 of the 1650 HCV-negative participants had HCC, the ROC curve in the HCV-negative group is less reliable. We measured the serum TGF- β 1 concentrations in the participants only once, at the time of the baseline survey. Therefore, we did not monitor chronological changes in the serum TGF- β 1 concentrations in relation to the HCC mortality rate. Although the self-administered questionnaire completed at the time of the baseline survey asked the participants whether they had any liver disease, we did not have any data regarding the medical histories of specific diseases, such as hepatic cirrhosis.

In summary, our nested case-controlled study of 1940 participants in the real-world setting without HCC at baseline displayed a strong association between a low serum TGF- β 1 concentration and a higher risk of incidence HCC. The effect size was substantial and preserved throughout different statistical assumptions and patient populations. Our inferences indicate that serum TGF- β 1 can distinguish between patients who are more likely to develop HCC from those with lower risk, especially among HCV-positive patients. TGF- β 1 may serve as the serum biomarker that predicts the development of malignancy even when clinically undetectable.

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Conflict of Interest

The authors have no conflicts of interest to disclose.

Author Contribution

YW did the statistical analysis and wrote the first draft. AI and YJS did the systematic literature search for the Research in Context section and the data interpretation, and critically revised the manuscript. All the authors were involved in the study design and approved the final manuscript.

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